Ozone potentiates vitamin E depletion by ultraviolet radiation in the murine stratum corneum

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Abstract As the outermost layer of the skin, the stratum corneum is exposed to environmental oxidants. To investigate putative synergisms of environmental oxidative stressors in stratum corneum, hairless mice were exposed to ultraviolet radiation (UV) and ozone (O_3) alone and in combination. Whereas a significant depletion of α-tocopherol was observed after individual exposure to either a 0.5 minimal erythemal dose of UV or 1 ppm O_3 for 2 h, the combination did not increase the effect of UV alone. However, a dose of 0.5 ppm $O_3 \times 2$ h, which had no effect when used alone, significantly enhanced the UV-induced depletion of vitamin E. We conclude that concomitant exposure to low doses of UV and O_3 at levels near those that humans can be exposed to causes additive oxidative stress in the stratum corneum.

Key words: Reactive oxygen species; Antioxidant; Malondialdehyde; Peroxidation

1. Introduction

Skin as an interface between the body and the environment is chronically exposed to oxidative stress from both ultraviolet (UV) radiation and environmental oxidant pollutants. It has been long recognized that UV-B, and to a much lesser extent UV-A, induce a number of pathological conditions in the skin, including erythema, edema, hyperplasia, sunburn cell formation, photoaging and photocarcinogenesis [1,2]. There is abundant information that reactive oxygen species (ROS), such as singlet oxygen, peroxy radicals, superoxide anion, and hydroxyl radical, are involved in UV-induced insults on the skin [1], both by direct effects of UV and by subsequent phagocyte infiltration and activation [2].

Oxidative environmental pollutants such as cigarette smoke, ozone (O_3) and oxides of nitrogen, which have been studied with regard to their effects on the respiratory tract [3], represent another potential oxidant stress to the skin. O_3 represents one of the major oxidative pollutants in photochemical smog, levels being frequently highest in heavily polluted areas where exposure to UV is also high. Cities located in air basins such as Los Angeles or Mexico City frequently are exposed to O_3 levels above the US federal safety standard of 0.12 ppm × 1 h, occasionally peaking at levels above 0.5 ppm [4]. We have recently documented that exposure of skin to as little as 1 ppm $O_3 \times 2$ h depletes the outermost skin surface tissue, the stratum corneum (SC), of low molecular weight antioxidants [5], which in themselves are present in SC in decreasing concentrations toward the skin surface, suggesting a degree of oxidative stress by ambient O_2 exposure [6].

Although exposure of cutaneous tissues to either UV [7] or O_3 [8] alone is known to deplete vitamin E and induce lipid peroxidation, there are no data characterizing possible additive effects of sequential or simultaneous exposure of skin to these important environmental oxidants stresses. The aim of the present study was to determine the effects of subsequent exposure to O_3 and UV radiation on SC α-tocopherol, and on markers of lipid peroxidation.

2. Materials and methods

2.1. Chemicals

All chemicals used were of the highest grade available. Authentic α-tocopherol standards were a gift from BASF (Ludwigshafen, Germany).

2.2. Animals

The animal care, handling, and experimental procedures were carried out in accordance with the protocol approved by Animal Care and Use Committee of the University of California, Berkeley. Hairless mice (females, between 7 and 10 weeks old, Charles River Laboratories, Wilmington, MA, USA) were kept under standard light and temperature conditions. Food (Harlan Tekland Rodent Diet 1846, WI, USA) and water were provided ad libitum. Mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight, Nembutal, Abbott Laboratories, North Chicago, IL, USA) and remained anesthetized during the entire exposure and sampling procedures.

2.3. O_3 exposure

O_3 was produced from O_2 by electrical corona arc discharge (Sander Ozonizer model IV, Eltze, Germany). The O_2–O_3 mixture was then mixed with ambient air and allowed to flow into an exposure chamber at a constant rate (200 l/min). The O_3 concentration in the exposure chamber was adjusted to 0.5 or 1.0 ppm and continuously monitored with an O_3 detector (Dasibi model 1003-AH, Glendale, CA, USA).

The O_3 chamber consisted of a teflon lined polyethylene formed in a cuboid shape with an inlet on one end and an outlet on the other end. Two oval holes of approximately 8 by 4 cm were cut in the flat roof of the chamber. A mouse was placed sideward on each hole, so

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Abbreviations: SSUV, solar simulated ultraviolet; ROS, reactive oxygen species; MDA, malondialdehyde; SC, stratum corneum; MED, minimal erythemal dose; HPLC, high performance liquid chromatography

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that the right side of the murine back was exposed to the inside of the chamber. The weight of the mouse sealed the hole airtight. The O_3 concentrations on top of a sealed hole were not different from the ambient O_3 levels in the laboratory air. In this way a continuous and defined ozone exposure to one side of the murine dorsum could be achieved, while the other side remained untreated ($n=8$ for each experiment). Subsequent to O_3 exposure, the lower back only was subjected to UV radiation.

2.4. O_2 and air exposure

It is noteworthy that exposure to O_3 with this set up entails important factors: the concentration of O_3, the presence of O_2, and the continuous flow of air. One objective of the current study was to dissect out the contributions of these parameters. In addition to O_3 exposure, each experiment was carried out with air flow only ($n=4$), to determine the contribution of a continuous dry air current on the concentrations of SC substances. The O_2 which is not converted to O_3 accounts for more than 90% of the O_2 used for the generation of O_3. Therefore, controls where included where the ozonator was turned off, but the O_2 was added to the gas mixture ($n=4$).

2.5. UV irradiation

UV light was generated by a solar UV simulator (Oriel 100-W, Oriel, Stratford, CT, USA) using an atmospheric filter (filters <290 nm) to achieve a solar simulated UV (SSUV) spectrum, as previously described [7]. The irradiance at 310 nm (UV-B range) was 2.8 mW/cm^2 and 360 nm at 4.2 mW/cm^2 (UV-A range) as measured with a UV-X radiometer (UVP Inc., San Gabriel, CA). One murine minimal erythemal dose (MED) was obtained in 3 min of exposure time, corresponding to a SSUV dose of 1.26 J/cm^2 as measured by the addition of the radiometer readings. Since the integrals of the radiometer probes are fairly broad and overlap considerably, this value is somewhat overestimated. Upon completion of the O_3 exposure, the lower two quadrants of the mouse backs were irradiated for 60 or 90 s, corresponding to 0.33 and 0.6 MED, while the upper back was protected from UV light with protective shielding. This set-up allowed for four differently treated quadrants on the mouse back: one untreated part (upper left) and one O_3-treated quadrant on the upper back (upper right), one UV (lower left), and one UV plus O_3-treated quadrant (lower right) on the lower back. In this way, each mouse served as its own control. Preliminary experiments were carried out to evaluate the minimal dose of UV necessary to induce a significant depletion of α-tocopherol. As shown in Fig. 1, 0.1 MED had no effect on murine SC vitamin E concentration. However, exposure to 0.3 or 0.5 MED resulted in a 60% or 80% depletion of vitamin E respectively. Therefore, mice were exposed in all further experiments at a dose of at least 0.3 MED.

2.6. Tape stripping

Samples of murine SC were obtained by sequential removal of SC layers with adhesive discs (3.8 cm^2, D-SQUAME, Cuderm, Dallas, TX, USA). Each quadrant of the mouse back was tape stripped six times using a standardized protocol as described in greater detail previously [5]. The SC containing tapes from each quadrant were pooled and stored on dry ice until extraction for no longer than 30 min.

2.7. Vitamin E analysis

Vitamin E was extracted as described previously [5]. Briefly, the adhesive tapes containing the SC were extracted with 2 ml of phosphate buffered saline (1 mM EDTA), 50 μl butylated hydroxytoluene (0.10 g per 100 ml of absolute ethanol), 1 ml sodium dodecyl sulfate solution (0.1 M) and 4 ml of HPLC grade ethanol. The lipids were then extracted with hexane and taken to dryness under N_2, after which they were resuspended in ethanol/ methanol (1:1) for HPLC analysis. The HPLC system (Shimadzu, Kyoto, Japan) consisted of a SIL-10A autoinjector, a LC-10-AD pump, an ultrasphere ODS C18, 4.6 mm internal diameter, 25 cm long, 5 μm particle size column (Beckman, Fullerton, CA, USA). α-Tocopherol was eluted with methanol:ethanol 1:3 (v/v) containing 20 mM lithium perchlorate, and quantified with an amperometric electrochemical detector (LC-4B, BAS, West Lafayette, IN, USA). Other forms of vitamin E, such as γ-tocopherol, are present in only low concentration relative to α-tocopherol, and are below the detection limit of the tape stripping based sampling method of murine SC in the current configuration.

2.8. HPLC-based TBA assay

An aliquot of the ethanol extract from the vitamin E extraction was used for analysis of TBA-MDA adducts in a modification of standard protocols [9,10]. It must be noted that this assay does not exclusively measure the MDA formed by lipid peroxidation. Also other substances such as DNA, carbohydrates and amino acids can generate a true TBA-MDA adduct upon heating [11]. The chloroform extract of the sample was collected, dried under nitrogen, resuspended in 400 μl SDS (0.15 g/100 ml) and incubated with 250 μl TBA reagent (0.375 g TBA/100 ml) and 200 μl phosphoric acid (1.22 M) for 30 min at 100°C. Then, 380 μl methanol was added for protein precipitation and 20 μl 1 N NaOH for pH neutralization. After centrifugation, 100 μl of clear supernatant was injected into a HPLC system, consisting of a 114 M Solvent Delivery Module pump (Beckaman, Fullerton, CA, USA), an Alltima C18 5 μm, 250 mm × 4.6 mm internal diameter, reversed phase column (Alltech, Deerfield, IL, USA), and a Hitachi (Hitachi, Tokyo, Japan) F-105 fluorescence spectrophotometer. The mobile phase consisted of 60% methanol and 40% 50 mM NaH_2PO_4, pH adjusted to 5.5. The flow rate was 0.9 ml/min and the detector was set at excitation 532 nm and emission 553 nm. Samples and standard were analyzed in duplicate.

2.9. Statistics

Statistical analysis was carried out using InStat for Macintosh (Graphpad Inc., San Diego, CA, USA). For comparison of parameters obtained from the same mouse, a one factor repeated measure ANOVA analysis was performed, for data derived from different mice a single measure ANOVA was utilized. The Tukey post-test was used to determine differences between groups. To optimize the analysis, the values obtained from the untreated quadrant were used as a reference. The data of the other three quadrants were expressed as a ratio to the untreated reference and expressed as percent of control (data point × 100/untreated data point) throughout. All data are expressed as mean ± S.D. Generally, a $P<0.05$ is considered statistically significant.

3. Results

3.1. O_3 potentiates UV-induced depletion of vitamin E

The average content of α-tocopherol in the first six pooled tape strippings was 1.09 ± 0.17 pmol. In accordance with earlier findings [8], an O_3 dose of 1 ppm × 2 h significantly depleted SC vitamin E to 40.4 ± 9.0% of the values in untreated skin ($P<0.001$, Fig. 2). 0.5 MED of SSUV further depleted O_3-pretreated SC vitamin E to 19.4 ± 8.6% of the untreated SC ($P<0.01$). However, the combined effect of O_3 and UV radiation (19.6 ± 9.3%) was not more pronounced than UV treatment (0.5 MED) alone.

Since the effect of UV radiation was more evident as com-

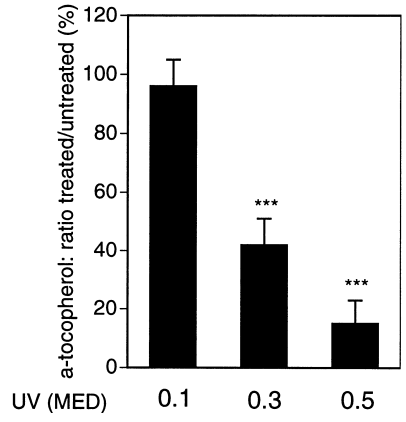

Fig. 1. SC α-tocopherol is depleted by SSUV. Four quarters of mice were exposed to increasing levels of UV radiation ($n=4$), and the depletion of SC α-tocopherol was monitored (***$P<0.001$).

pared to O_3 treatment, the experiment was repeated with lower doses of SSUV (0.33 MED) and 0.5 ppm $O_3 \times 2$ h. With the lower doses, an additive effect of the two stressors could be observed. UV radiation with only 0.33 MED significantly depleted α-tocopherol to 45.0 ± 13.7% of the untreated control site. O_3 treatment with 0.5 ppm × 2 h alone did not significantly decrease α-tocopherol (66.4 ± 21.0%) as compared to O_2-treated controls (83.3 ± 4.0%). When this insignificant dose of O_3 was used as a pretreatment to low dose UV radiation (0.33 MED), the α-tocopherol depletion was enhanced to 20.0 ± 7.9% of the levels in untreated SC (Fig. 2). This additive effect of combined stressors was significant when compared to either O_3 treatment (0.5 ppm × 2 h, $P < 0.001$) or UV treatment (0.33 MED, $P < 0.01$) alone.

3.2. Effect of O_3 as opposed to O_2 or air

To demonstrate that the additive effect of O_3 and UV radiation was not an artifact of the oxidizing effect of O_2, which was present in the gas mixture used for O_3 treatment, the experiments described above were carried out using only ambient air with or without added O_2 in the gas mixture. Treatment with ambient air under a flow of 200 l/min showed a trend to increase the vitamin E concentration in SC to 122 ± 9.0% of the controls, while the additional presence of added O_2 in the gas phase significantly decreased vitamin E to 86.4 ± 8.8%. This effect was significant when compared to air only treatment ($P < 0.01$). When using a UV dose of 0.5 MED, pretreatment with O_2 failed to further deplete vitamin E (20 ± 9.3% for UV and 22 ± 8.2% for UV plus O_2). Even when a low UV dose of 0.33 MED was used, the presence of O_2 in the pretreatment did not result in an additive depletion of α-tocopherol (42 ± 5.0% for UV and 48 ± 9.0% for combined treatment) (Fig. 3).

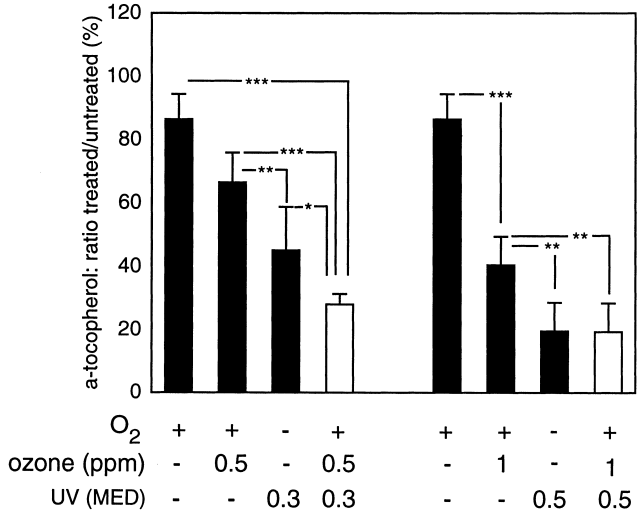

Fig. 2. A low dose of O_3 increased the UV-induced depletion of SC α-tocopherol. After UV and O_3 treatment, SC were obtained by tape stripping (six consecutive strips pooled), and α-tocopherol concentrations were analyzed. Remarkably, α-tocopherol was strongly depleted by high doses of O_3 (1 ppm × 2 h) and UV (0.5 MED), and the combined effect of O_3 and UV radiation was not more pronounced than UV treatment alone. In contrast, the additive effect of combined stressors was significant with low concentrations of UV (0.3 MED) and O_3 (0.5 ppm × 2 h) when compared to either O_3 treatment or UV treatment alone (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$).

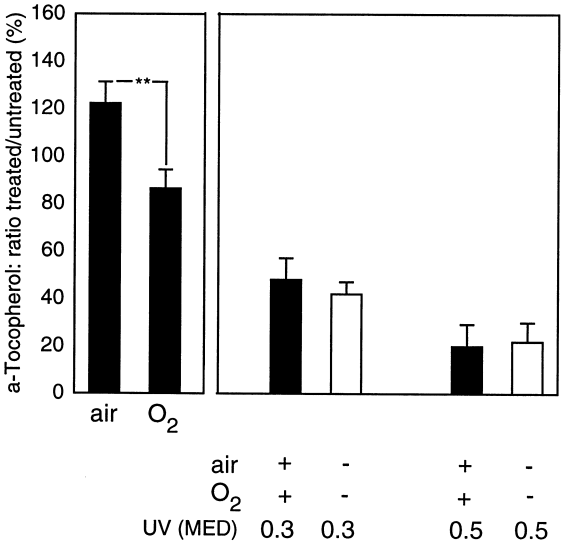

Fig. 3. O_2 does not affect the UV-induced depletion of SC α-tocopherol. The experiments shown in Fig. 2 were repeated with O_2 instead of O_3. O_2 did not cause a significant decrease in vitamin E as compared to untreated control (**$P < 0.01$).

3.3. O_3 effect on UV-induced oxidative damage to SC lipids

The MDA-TBA adduct, a marker of lipid peroxidation, was analyzed after different treatment conditions. The baseline concentration in extracts from six pooled tape strips acquired from untreated SC was 12.65 ± 1.1 pmol. Exposure to a flow of air (200 l/min) showed an insignificant trend to increase MDA baseline levels to 112 ± 16.4% of untreated SC. This is most likely due to a drying effect caused by the continuous air flow. The presence of additional O_2 in the air mixture did not affect the MDA content (115 ± 21.6%) as compared to untreated or air-treated SC.

However, 0.5 MED of SSUV significantly increased MDA levels as compared to no treatment ($P < 0.001$) or air ($P < 0.001$) or air plus O_2 treatment ($P < 0.001$). Neither a lower dose of UV (0.33 MED, 134.8 ± 1.08%) nor a dose of 0.5 ppm $O_3 \times 24$ h (121.3 ± 5.7%) changed the MDA levels significantly. Also, a combination of the two oxidants did not increase the MDA content (126.0 ± 20.0%).

4. Discussion

The present study clearly shows that O_3 concentrations at levels that are occasionally present in areas highly polluted with photochemical smog can enhance UV-induced vitamin E depletion in outermost cutaneous tissues. We conclude that ambient exposure to O_3 and UV radiation is capable of causing an additive oxidative stress to exposed cutaneous tissues.

The additive effect of UV radiation and O_3 was most prominent with lower doses of both environmental oxidants. With higher levels of both oxidants, UV radiation still increased the O_3-induced vitamin E depletion. 1 ppm $O_3 \times 2$ h, a concentration slightly above the maximum recorded during smog situations [4], depleted α-tocopherol in accordance with earlier findings [8]. However, this dose was not sufficient to enhance the UV effects, even though the doses of SSUV were still well below the level where erythema responses could be observed

in our experiments. Not surprisingly, UV radiation appears to pose a considerably higher oxidative stress to cutaneous tissues than environmental O_3 exposure.

The fact that lipid peroxidation was only increased when at least 0.5 MED SSUV were used, whereas vitamin E loss was seen at lower levels, adds further evidence of the role of vitamin E as a sacrificial and chain breaking antioxidant in the prevention of progressive lipid peroxidation in cutaneous tissues. This is consistent with many studies showing that lipid peroxidation does not occur until vitamin E is almost completely depleted [12]. It can be speculatively concluded that initially the SC antioxidant defenses are sufficient to prevent oxidative damage to lipids, and that in the SC vitamin E depletion may be a more sensitive marker of oxidative stress than MDA formation.

UV radiation and O_3 may deplete vitamin E by multiple pathways. Since SSUV contains a portion of UV-B it is able to directly destroy α-tocopherol, which absorbs in the lower UV-B range (295 nm) [13]. Through the process of photosensitization UV can directly oxidize target molecules (type I) or can transfer its energy to O_2 (type II) producing ROS [14]. For example, the UV-A portion is involved in the production of singlet oxygen. These species can either react with vitamin E directly or cause lipid peroxidation cascades, which eventually consume vitamin E. In a similar fashion O_3 is known to directly react with vitamin E [4,15] or can induce the formation of ROS such as H_2O_2 [16,17]. Lipids may be destroyed directly via ozonization [16] or ROS. Vitamin E then could be depleted either by lipid peroxidation processes or by the scavenging of ROS.

While UV radiation penetrates into the epidermis (UV-B) or into the dermis (UV-A), and is known to induce the release of tissue degrading enzymes even at suberythemal levels [18], O_3 oxidizes biological systems only at the surface [16,19] Therefore, even though it is demonstrated that O_3 and UV cooperatively damage SC components, it is not yet known if they can exert additive effects in the deeper layers of the epidermis. In the lung, O_3 toxicity is believed to be mediated by secondary bioactive lipid oxidation products such as aldehydes [16]. By analogy, products of O_3-induced lipid oxidation may penetrate the outer skin barrier and cause effects to constituents of the deeper epidermis. In the lung, the activation of NF-κB by O_3 has been demonstrated [20,21]. NF-κB activation has also been implicated in the expression of collagenases by solar simulated UV radiation [18,22] and in cutaneous responses to wounding [23].

UV radiation has been shown to compromise the skin barrier [24]. Although not shown in the current study, O_3 may enhance this phenomenon by perturbing SC lipid constituents in the SC, which are known to be critical determinants of the barrier function [25]. Thus, O_3 may cause a disturbance of the barrier function, increase the transepidermal water loss and provoke epidermal repair responses [20], as can be also seen after barrier perturbation [26].

UV radiation is a causal factor in both premature skin aging (photoaging) and skin carcinogenesis [1]. Oxidative processes are believed to be critical mediators of those events [27]. Since O_3 enhances UV-induced oxidation in the SC, it cannot be excluded that potentially O_3 also enhances other UV effects such as photoaging.

In conclusion, this study demonstrates that O_3 and UV radiation, two common sources of environmental oxidant stressors, when presented at appropriate doses, exhibit additive effects in terms of oxidative damage to the murine skin barrier. Further studies are warranted to analyze the interaction of those two oxidants with other epidermal constituents and in skin pathologies involving oxidative damage.

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